Liquid Chromatography; Liquid Chromatography-Nuclear Magnetic Resonance; Supercritical Fluid Extraction; Thin Layer (Planar) Chromatography. **Terpenoids: Liquid Chromatography.**

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Liquid Chromatography

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Chemical investigation of plant constituents is strongly linked to the use of liquid chromatography (LC) at both the analytical and preparative level. Indeed, most of the secondary metabolites can be efficiently separated or isolated by different liquid chromatographic techniques. The plant extracts are generally screened by different bioassays and submitted to fractionation by chromatography. The fractions obtained are further tested for their biological activities. This process is repeated until the isolation of a pure active constituent, which is finally identified by spectroscopic methods (bioactivity guided isolation; **Figure 1**).

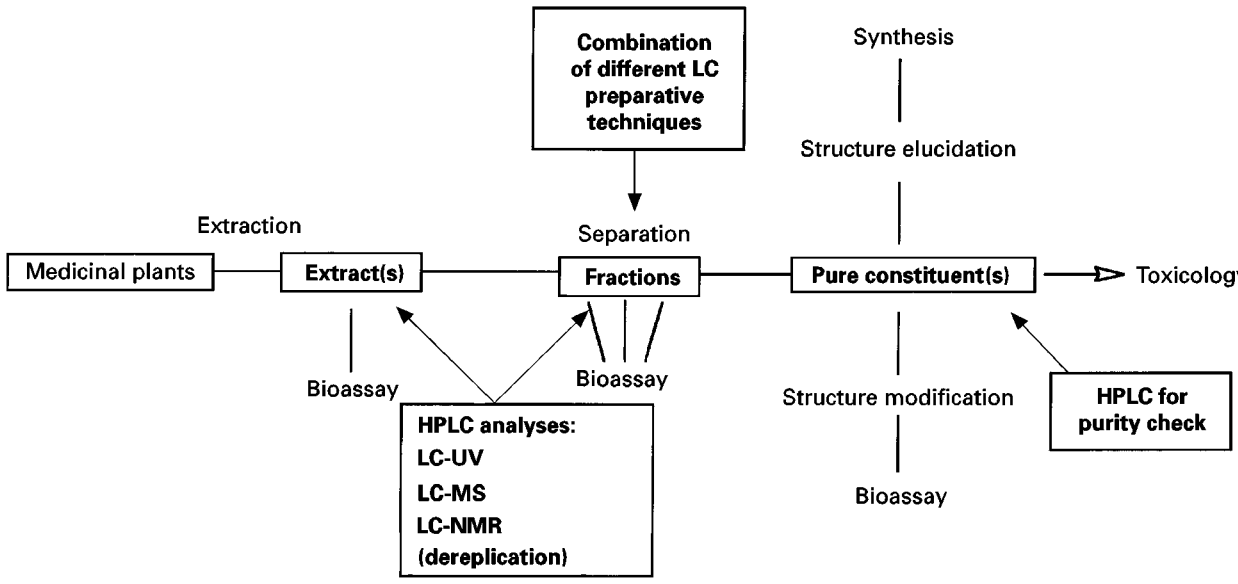

Figure 1 Procedure for obtaining the active principles from plants. LC techniques are used at the analytical and preparative level during the whole isolation procedure. HPLC hyphenated techniques play an important role in the early recognition of well-known compounds in the extract prior to isolation.

Generally the plant material is extracted by solvents of increasing polarity. This extraction step is very important because it allows a first rough fractionation of the plant constituents. Initial extraction with low polarity solvents yields the more lipophilic components, while alcoholic solvents give a larger spectrum of apolar and polar material. The plant extracts are usually very complex mixtures which contain hundreds or thousands of different constituents. Separation of all these constituents with a single chromatographic technique is often difficult to achieve. Thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) are the most commonly used techniques for a rapid check of the chemical composition of these extracts. If the isolation of a given constituent is required, a scale-up of the analytical separation conditions to preparative chromatography techniques is needed. These preparative techniques are generally open-column chromatography, low pressure LC (LPLC), medium pressure LC (MPLC) and semi-preparative HPLC. When irreversible adsorption problems or denaturation of natural products occur, countercurrent chromatography (CCC) techniques such as centrifugal partition chromatography (CPC) are preferred.

Analytical Techniques

High Performance Liquid Chromatography

Of all the LC chromatography techniques used in the analysis of plants, HPLC is probably the most useful and versatile. HPLC is used routinely in phytochemistry to pilot the preparative isolation of natural products (optimization of the experimental conditions, checking of the different fractions throughout the separation) and to control the final purity of the isolated compounds. For chemotaxonomic purposes, the botanical relationships between different species can be shown by chromatographic comparison of their chemical composition. Comparison of chromatograms, used as fingerprints, between authentic samples and unknowns permits identification of the unknown material and/or the search for adulteration.

HPLC of complex mixtures such as crude extracts is usually carried out on reversed-phase columns. For the separation of the different constituents, acetonitrile–water or methanol–water gradients are applied. Linear gradients are generally used but in the case of a very complex separation, a succession of isocratic and gradient steps may be necessary. For the suppression of tailing due to polyphenolic constituents, modifiers such as trifluoroacetic acid or acetic acid are added, while for the separation of basic products such as alkaloids, amines or NH_4OH can be used. Crude extracts or fractions are usually injected without any sample preparation, but solid-phase extraction (SPE) prepurification or derivatization (usually to enhance the UV chromophore) can be performed in certain cases. The sample is preferably dissolved in the eluent but when solubility problems occur, as is often the case with plant extracts, stronger solvents such as tetrahydrofuran are used. When working with 4 mm i.d. columns, 50–200 μg of extract can be injected. In most cases, the separation is usually followed by UV detection because a majority of natural products possess UV chromophores. However, for the detection of constituents without a UV chromophore such as sugars, other techniques such as refractive index (RI), light scattering (LS) or electrochemical detection (ECD) are necessary.

A typical HPLC-UV chromatogram of the crude methanol extract of the roots of the African plant *Chironia krebsii* (Gentianaceae) is shown in **Figure 2**. The separation was achieved by applying a linear acetonitrile–water gradient on an RP-18 column. A good separation of the three main classes of constituents found in the plant was achieved.

HPLC Hyphenated Techniques

In many applications, it may be necessary not only to detect but also to identify compounds in extracts. With conventional detection methods such as UV, the identity of peaks can be confirmed only from their retention times, by comparison with authentic samples. In order to get more information on the metabolites of interest, there is a need for a multiple detection system offering the possibility of taking advantage of both chromatography as a separation method and spectroscopy techniques as detection and identification methods. HPLC has thus been coupled to various sophisticated detectors such as UV photodiode array detectors (LC-UV-DAD), mass spectrometers (LC-MS) or, more recently, to nuclear magnetic resonance instruments (LC-NMR).

When searching plant extracts for compounds with interesting properties, a multidimensional approach to their chromatographic analysis is of great significance. By combining HPLC online with UV, MS and NMR, a large amount of preliminary information can be obtained about the constituents of an extract before their isolation (Figure 1). In the case of polyphenols, for example, UV spectra recorded online give useful complementary information (type of chromophore or pattern of substitution) to that obtained with LC-MS and already provide a precise assignment of the peak of interest. When these data are not sufficient, LC-NMR can give a useful

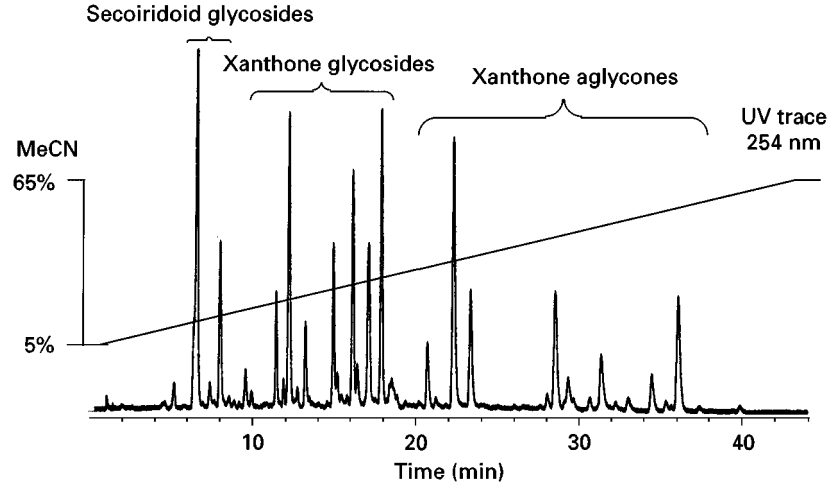

Figure 2 HPLC-UV analysis of the methanolic extract of the root of *Chironia krebsii* (Gentianaceae). HPLC: column, RP-18 NovaPak (4 μm, 150 × 3.9 mm i.d.); gradient, MeCN–H_2O (0.1% TFA) 5 : 95 → 65 : 35 in 50 min (1 mL min^{-1}).

complement for a full structural identification online. This preliminary LC chemical screening avoids the useless isolation of known constituents and concentrates the search only on the compounds of potential further interest.

This approach is illustrated by the following example: a Gentianaceous plant, *Swertia calycina*, presenting interesting antifungal activities, was studied by LC-UV, LC-MS and LC-NMR. The LC-UV analysis of the dichloromethane extract was quite simple and exhibited three main peaks. The LC-UV spectra of these peaks permitted a first rapid online recognition of the various types of constituents of the extract. The LC-MS provided molecular weight information for each of these compounds (**Figure 3**).

These online data, together with chemotaxonomical considerations, allowed the identification of a xanthone (**3**) and a secoiridoid (**1**) in this plant. However, the structure of compound (**2**) could not be completely ascertained by LC-UV-MS alone, because its UV spectrum was characteristic for a quinonic chromophore and no compound of this type has been reported in the Gentianaceae family. The LC-MS of (**2**) exhibited a protonated $[M + H]^+$ ion at m/z 189, indicating a molecular weight of 188 amu. The LC-NMR analysis of the extract gave well resolved ^{1}H-NMR online spectra for the three major compounds. For the unknown (**2**), the online ^{1}H-NMR spectrum revealed the presence of a methoxyl group together with a quinonic ring proton while four other aromatic protons appeared at lower field (**Figure 4**). These online data were in good agreement with those of *O*-methyl lawsone, a known naphthoquinone. This type of compound has, however, never been reported in the Gentianaceae family.

Preparative Techniques

Preparative-scale separation is one of the most important operations carried out in a natural products laboratory. It is often tedious and time-consuming, especially when the mixture to be separated is complex. The nature of the separation problem varies considerably from the isolation of small quantities (mg or less), for structure determination purposes, to the isolation of very much larger amounts (hundreds of mg to kg quantities), for comprehensive biological testing or even for production of therapeutic agents.

The most important preparative techniques which have found application in the isolation and purification of natural products are listed in **Table 1**. A distinction has to be made between techniques using a solid stationary phase or a liquid fixed on an inert solid support and all-liquid partition techniques. For all these techniques, the use of solvents such as acetone or in some cases chloroform is inadvisable because in their presence natural products may undergo transformations.

Flash Chromatography/Open-column Chromatography

Open-column chromatography and flash chromatography are the most popular techniques for natural product isolation. Flash chromatography is a preparative air-driven liquid chromatographic technique with moderate resolution. It has the advantage over conventional open-column chromatography of minimizing sample loss and the risk of decomposition of natural products, due to the fast elution of the sample. These two techniques allow the use of various column sizes and permit the introduction of

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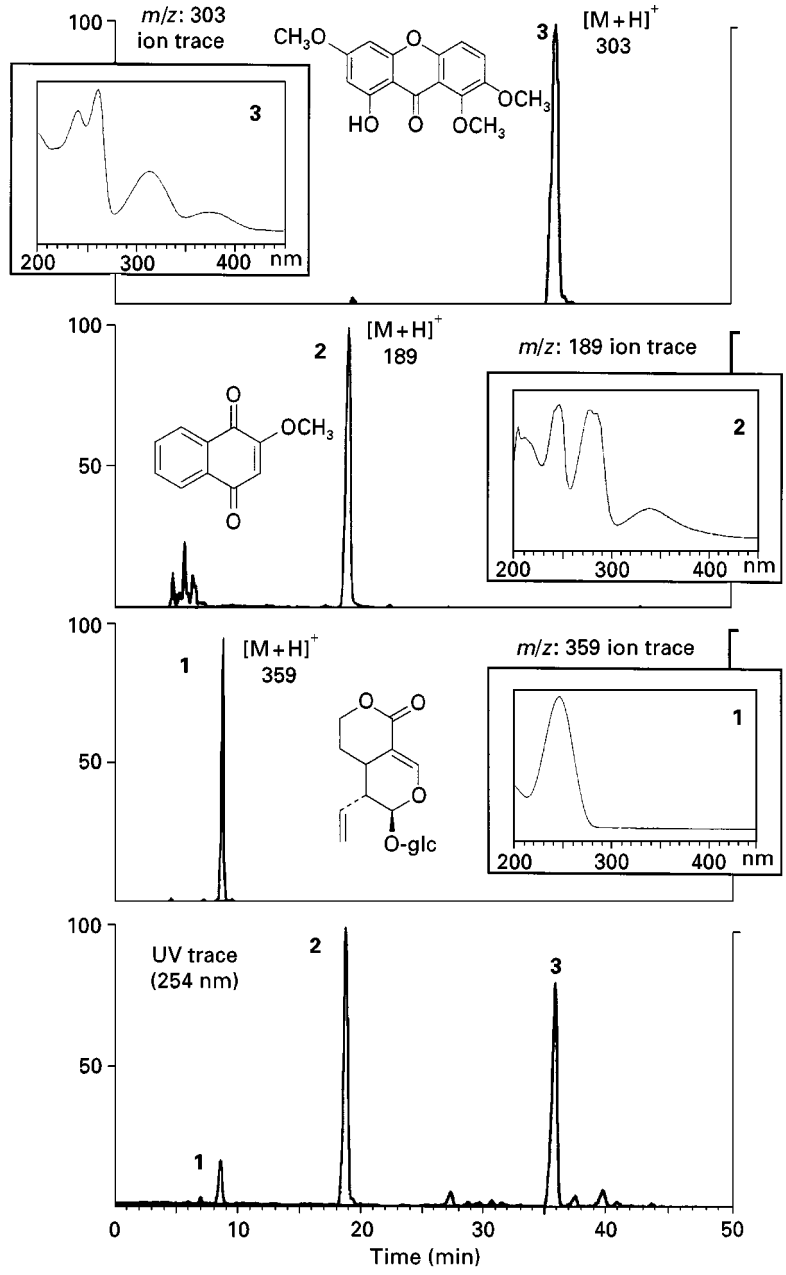

Figure 3 LC-UV and LC-thermospray (TSP)-MS analyses of the crude CH_2Cl_2 extract of *Swertia calycina* (Gentianaceae). For each major peak, the single ion LC-MS traces of the protonated molecular ions $[M + H]^+$ are displayed, together with the UV spectra obtained online.

mg to g quantities of sample. They are generally used with silica gel supports. As a rule of thumb, for natural product applications, around 30 mg sample loading per g of 50–200 μm support is feasible. When used only for filtration (first fractionation step of a crude plant extract), silica gel chromatography can be performed under overloaded conditions and quantities up to 100 mg g^{-1} of support can be loaded.

These techniques can be used at different steps in the isolation process of natural products. They are mostly well adapted to the separation of nonpolar to medium polarity plant extracts. In order to find the appropriate solvent system, preliminary TLC analyses are performed and the plate is sprayed with different reagents in order to detect the natural products of interest. For a given product, solvent systems producing R_F values between 0.2 and 0.3 are

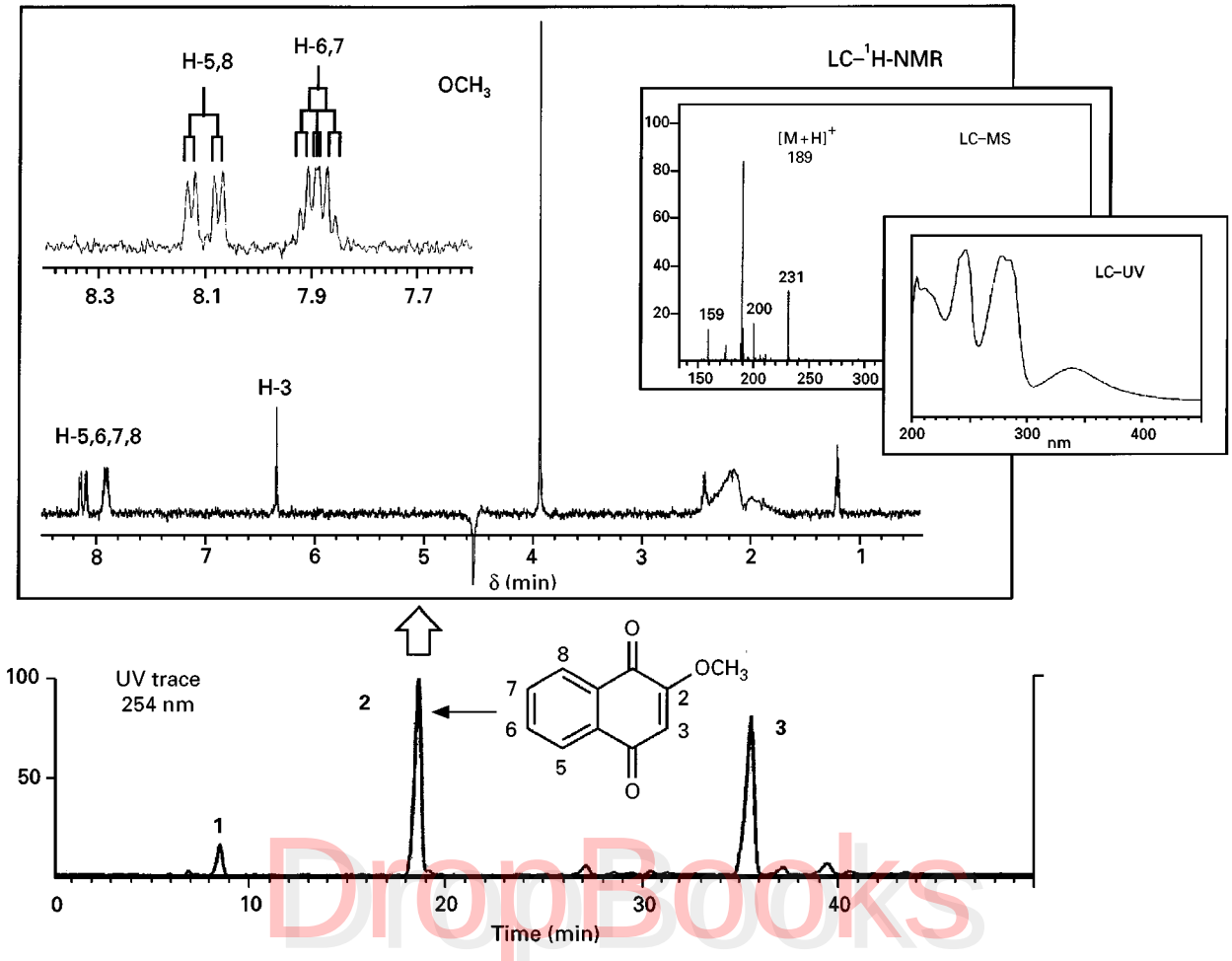

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Figure 4 Summary of all the spectroscopic data obtained online by LC-UV, LC-MS and LC-NMR for the naphthoquinone (**2**) in the dichloromethane extract of *Swertia calycina* (Gentianaceae).

usually optimum for scale-up. When flash and open-column chromatography are used as first fractionation steps of crude plant extracts, solvent systems of increasing polarity are often employed. The fractions are checked by TLC at the outlet of the column and when compounds of a given polarity are eluted, the solvent system is changed for a more polar one. This allows compounds of different polarities to be efficiently separated.

A typical step gradient solvent system for the separation of a dichloromethane extract by flash chromatography on silica gel is: chloroform–

Table 1 Preparative separation methods for plant constituents

Type	*Technique*	*Abbreviation*
Preparative thin-layer chromatography	Centrifugal TLC	CTLC
Open-column chromatography		CC
Vacuum liquid chromatography		VLC
Pressure liquid chromatography	Flash	
	Low pressure LC	LPLC
	Medium pressure LC	MPLC
	High performance LC	HPLC
Liquid–liquid chromatography	Droplet countercurrent chromatography	DCCC
	Centrifugal partition chromatography	CPC

petroleum spirit (80 : 20), then pure chloroform, chloroform–methanol (80 : 20) and finally pure methanol for eluting the polar constituents.

Preparative Liquid Chromatography

Because of the complexity of the chemical composition of crude plant extracts, open-column chromatography or flash chromatography techniques alone often do not provide sufficient resolution for the isolation of pure natural products. The introduction of pressurized liquid chromatography in natural product chemistry has allowed the use of silica gel of smaller particle size and of bonded phases such as RP-8, RP-18 and diol, thus giving more versatility and better resolution.

Depending on the sample size and the resolution to be achieved, three techniques are generally used for phytochemical applications: LPLC, MPLC and semipreparative HPLC. Polar natural products such as glycosidic derivatives can mainly be separated on RP-8 or RP-18 bonded phases while compounds of intermediate polarity such as polyphenol aglycones, polyacetylenes and sesquiterpenes can be resolved on silica gel supports (**Table 2**).

Low and Medium Pressure Chromatography

LPLC makes use of columns containing packings of *c.* 40–60 μm, allowing high flow rates up to a pressure of 10 bar. Prepacked columns with various sizes and supports are available. MPLC accommodates much larger sample loads (100 mg–100 g) than LPLC (10 mg–1 g) and is designed to be operated at higher pressure (*c.* 5–40 bar). MPLC uses refillable columns of various sizes. Supports with particle sizes ranging from 15 to 200 μm can be used. MPLC is more versatile than LPLC but the basic operation of the two techniques is similar.

The chromatographic conditions for these techniques are selected by HPLC or by TLC. The selectivity of the eluent is first optimized and then the composition of the mobile phase is adjusted to suit the preparative conditions. Retention factors (k) between 1 and 5 or R_F values < 0.4 are appropriate. Different representative solvent systems used for the separation of various classes of natural products are given in Table 2.

In some cases, similar resolution can be obtained by both LPLC or MPLC and analytical HPLC. This is of importance for the transposition of conditions from analytical to preparative level. It is illustrated here by the separation of iridoids and a phenylpropane glycoside from the root of *Sesamum angolense* (Pedaliaceae), a plant reputed to have antihaemorrhagic properties in African traditional medicine. Analytical HPLC of the relevant fractions is shown in **Figure 5A** and 5B. Baseline separation of phlomiol (**4**) and puchelloside-I (**5**) was possible with methanol–water (10 : 90). Verbascoside (**6**) was, however, only eluted with 32% methanol. These analytical conditions could be applied directly to a preparative separation on a Lobar LPLC column by performing a step-gradient elution from 10% to 32% methanol.

Following a similar procedure, several xanthone glycosides (**7–12**) from *Halenia corniculata* (Gentianaceae) were successfully isolated from an enriched fraction by MPLC in a single isocratic run (**Figure 6**).

Semipreparative HPLC

While analytical HPLC is useful for obtaining information about sample mixtures and does not rely on their recovery, the aim of semipreparative HPLC is to isolate pure substances. Compared to the other column LC techniques, HPLC can handle very small particle sizes (5–30 μm) which provide a large gain in separation efficiency. In phytochemical investigations, this technique is often used for final purification steps for a small number of compounds which are

Table 2 Representative solvent systems used for LPLC or MPLC

Substance class	*Support*	*Eluent*
Polyacetylenes	SiO_2	Toluene–EtOAc (85 : 15)
Flavonoid aglycones	SiO_2	C_6H_{14}–EtOAc, $CHCl_3$–MeOH
	DIOL	$CHCl_3$–MeOH–HOAc (950 : 50 : 1)
Flavonoid glycosides	RP-8	MeOH–H_2O gradient
Phenylpropanoid glycosides	RP-18	MeOH–H_2O gradient
Chromenes	SiO_2	$CHCl_3$–C_6H_{14}–MeOH
Sesquiterpenes	SiO_2	C_6H_{14}–EtOAc
Diterpenes	RP-18	MeOH–H_2O (1 : 1)
Triterpenes	RP-8	MeOH–H_2O (4 : 1)
Iridoid glycosides	RP-18	MeOH–H_2O gradient
Alkaloids	RP-8	MeOH–0.02 mol L^{-1} NH_4OAc (3 : 2)

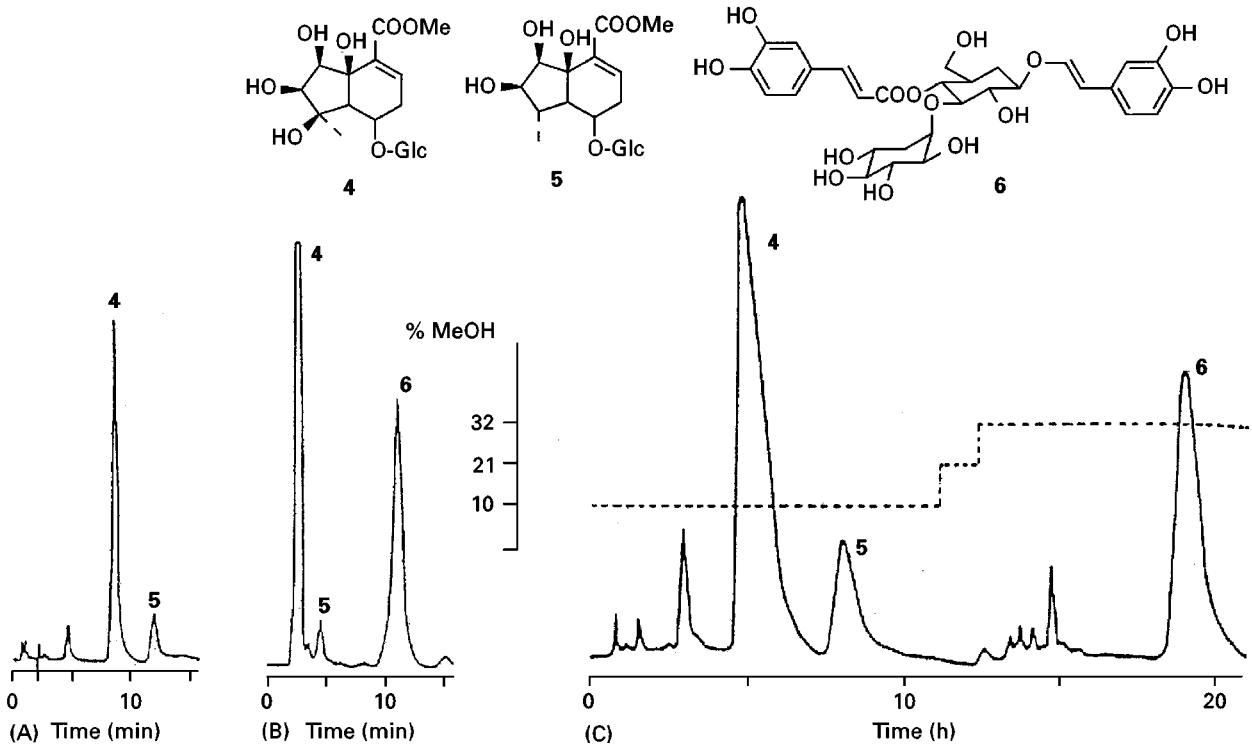

Figure 5 Isolation of iridoids from *Sesamum angolense*. (A) Analytical HPLC: column, LiChrosorb RP-8; eluent, MeOH–H_2O 10 : 90; detection, 254 nm. (B) Analytical HPLC: column, LiChrosorb RP-8 eluent, MeOH–H_2O 32 : 68; detection, 254 nm. (C) Preparative separation: column, Lobar RP-8 (310 × 25 mm i.d.); eluent, step gradient MeOH–H_2O 10 : 90 and 32 : 68; sample, 130 mg; detection, 254 nm.

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difficult to separate. The loading capacity of the column is usually rather small (1–100 mg) and the isolation of a pure natural product often requires multiple injections of a given prepurified fraction. The number of applications of this technique to natural product isolation is very large.

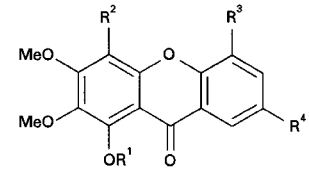
Figure 7 shows the separation of closely related antifungal chromenes from *Hypericum revolutum* (Guttiferae) by semipreparative HPLC. The petroleum ether extract of the leaves and twigs after flash chromatography and LPLC on silica gel, gave an active fraction which appeared homogeneous by TLC. However, analytical HPLC on an RP-18 column showed the presence of two homologues (**13** and **14**). Since chromenes degrade in the presence of acid, forming the corresponding dichromenes, no acid was used in eluent for semipreparative separation. A total of 120 mg was separated by repetitive injections.

Centrifugal Partition Chromatography

The use of CCC represents an interesting complementary approach to LC on solid supports. This technique is an all-liquid method without the presence of a solid support and thus has the advantage over other liquid chromatographic methods of avoiding irreversible adsorption of sample, allowing a quantitative recovery and minimizing the risk of sample denaturation. These points are extremely important when labile or polar natural products have to be isolated. Among the different countercurrent liquid chromatography techniques, CPC is probably the most popular technique because of its speed and ease of use. The choice of the solvent system can be guided by TLC and for rotating coils the best R_F range is 0.1–0.4. According to the type of instrument used – single coil, multiple coil or cartridge – the sample size ranges from 10 mg to 10 g. Selected examples of solvent systems used for the separation of various classes of natural products are given in **Table 3**.

In order to illustrate this approach, the separation of the active anthranoid pigments of *Psorospermum febrifugum* (Guttiferae), an African medicinal plant that exhibits strong growth inhibition of cancer cells and antimalarial activity, is shown in **Figure 8**. The lipophilic root bark extract of this plant was first separated by flash chromatography and LPLC but resulted in considerable material losses, owing to irreversible adsorption on the supports. However, in a single CPC step, three pure compounds (**15**, **16** and **17**) and a mixture of a fourth anthranoid pigment

	R^1	R^2	R^3	R^4
7	Gent		OMe	
8	Prim		OMe	
9	Prim	OMe	OMe	
10	Prim		OMe	
11	Prim	OMe		OMe
12	Prim	OMe	OMe	OMe



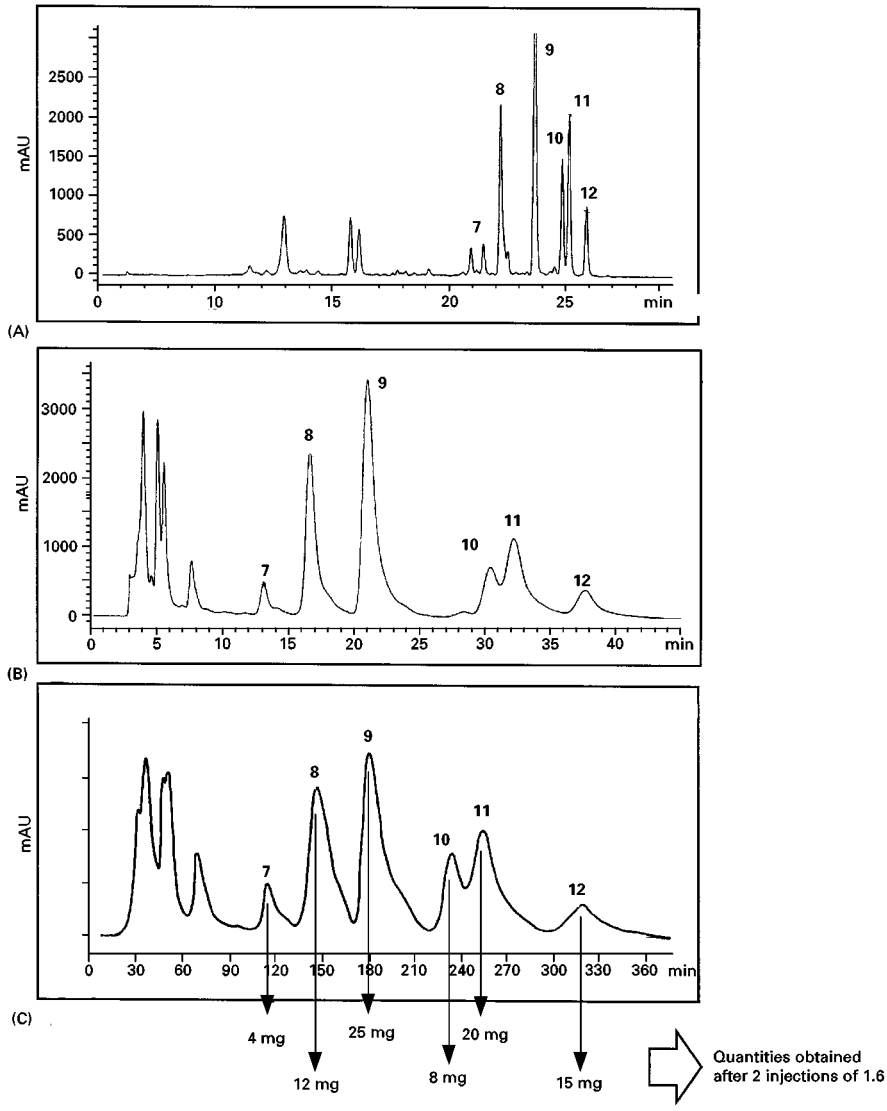


Figure 6 HPLC-MPLC transposition for the isolation of xanthone glycosides from an enriched fraction of *Halenia corniculata* (Gentianaceae) after separation by size exclusion chromatography on Sephadex LH-20 of the methanolic extract. (A) HPLC-gradient. Conditions: column: NovaPak RP-18 (3.9 × 150 mm); H_2O–MeCN; 5–65% MeCN in 50 min; 1 mL min^{-1}. (B) HPLC-isocratic. Conditions: column: LiChroCart; Lichrosorb RP-18 (7 μm); 4 × 250 mm; H_2O–MeOH (60 : 40); 1 mL min^{-1}. (C) MPLC-isocratic. Conditions: column: MPLC home-packed Lichrosorb Rp-18 (15–25 μm); 12 × 460 mm; H_2O–MeOH (60 : 40); 3 mL min^{-1}; 12 bar. Sample size 300 mg.

(**18**) with an unidentified constituent (**19**) were obtained without loss of product. A nonaqueous solvent system was used for the separation, which could be scaled up to a 500 mg sample size.

Combination of Methods

No single liquid chromatographic separation method is able to solve all separation problems and moreover

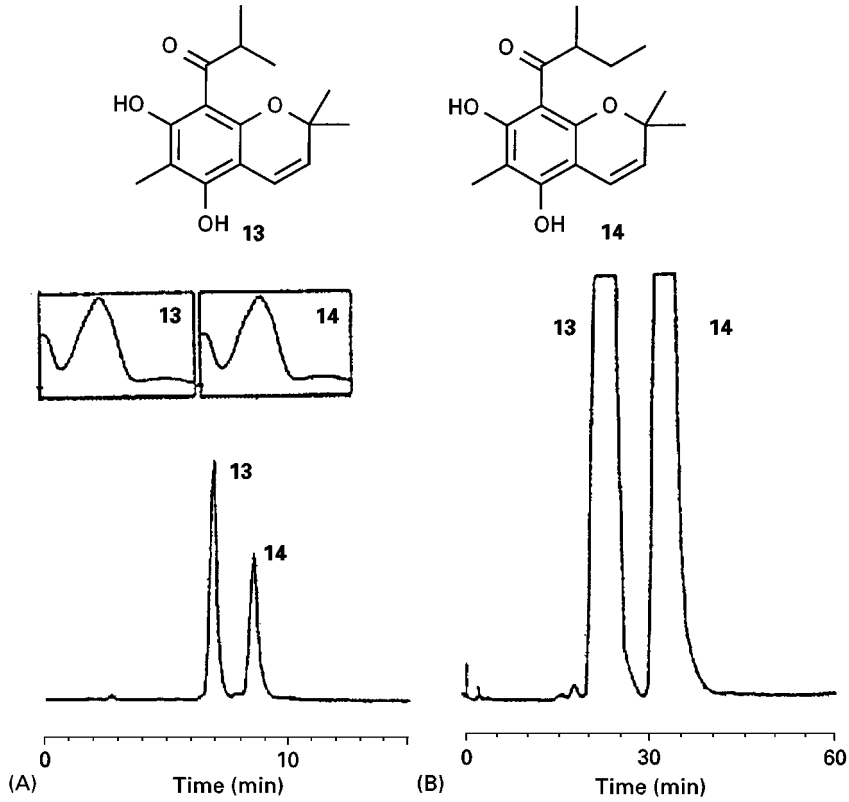


Figure 7 Separation of antifungal chromenes from *Hypericum revolutum* (Guttiferae). (A) Analytical HPLC: column, LiChrosorb RP-18 (250 × 4.6 mm i.d.); solvent, MeOH–H_2O 80 : 20; flow, 1.5 mL min^{-1}; detection, 254 nm. (B) Semipreparative HPLC: column, μ-Bondapak RP-18 (300 × 7.8 mm i.d.); solvent MeOH–H_2O 67 : 37; flow, 5 mL min^{-1}; detection, 254 nm.

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it is very common to find multistep chromatographic operations for the isolation of pure natural products. Although it is possible to obtain a pure compound by a one- or two-step procedure, a combination of techniques is normally required. Of course there are many different ways of putting together all the possible separation techniques, but in reality the choice of strategy is limited

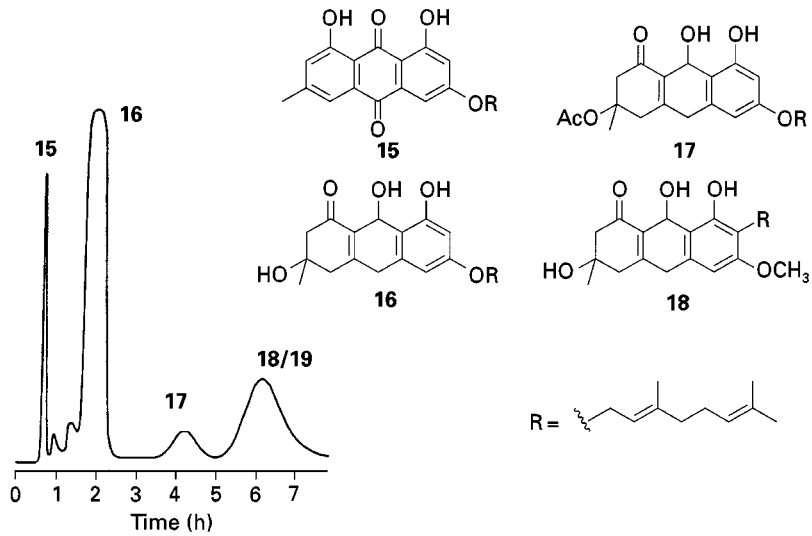

Figure 8 CPC separation of a light petroleum ether extract of *Psorospermum febrifugum* (Guttiferre) root bark. Solvent, *n*-C_6H_{14}–MeCN–MeOH (40 : 25 : 10, mobile phase = upper phase); flow, 5.5 mL min^{-1}; rotational speed, 1500 rpm; sample, 100 mg; detection, 254 nm.

Table 3 Representative solvent systems used for CPC separations

Substance class	*Eluent*
Flavonoids	$CHCl_3$–MeOH–H_2O 4 : 3 : 2
Xanthones	Petrol ether–EtOAc–MeOH–H_2O 1 : 1 : 1 : 1
Tannins	*n*BuOH–*n*PrOH–H_2O 4 : 1 : 5
	*n*BuOH–*n*PrOH–H_2O 2 :1 : 3
Saponins	$CHCl_3$–MeOH–*n*PrOH–H_2O 5 : 6 : 1 : 4
	$CHCl_3$–MeOH–*i*BuOH–H_2O 7 : 6 : 3 : 4
	$CHCl_3$–MeOH–H_2O 7 : 13 : 8
Polyacetylenes	Hexane–MeCN–TBME 10 : 10 : 1
Aporphine alkaloids	$CHCl_3$–MeOH–0.5% HOAc 5 : 5 : 3

by a number of constraints: the extraction method, the complexity of the extract, the sample preparation, the polarity, the stability, the solubility, the sample size and the complementarity of the separation techniques.

When choosing a separation strategy, it is often useful to pick steps which differ as much as possible in selectivity. During an isolation procedure, the scale of the operation decreases: as the purity of the product increases, there is a corresponding diminution of sample quantity. This implies that the initial fractionation steps are those which can separate large

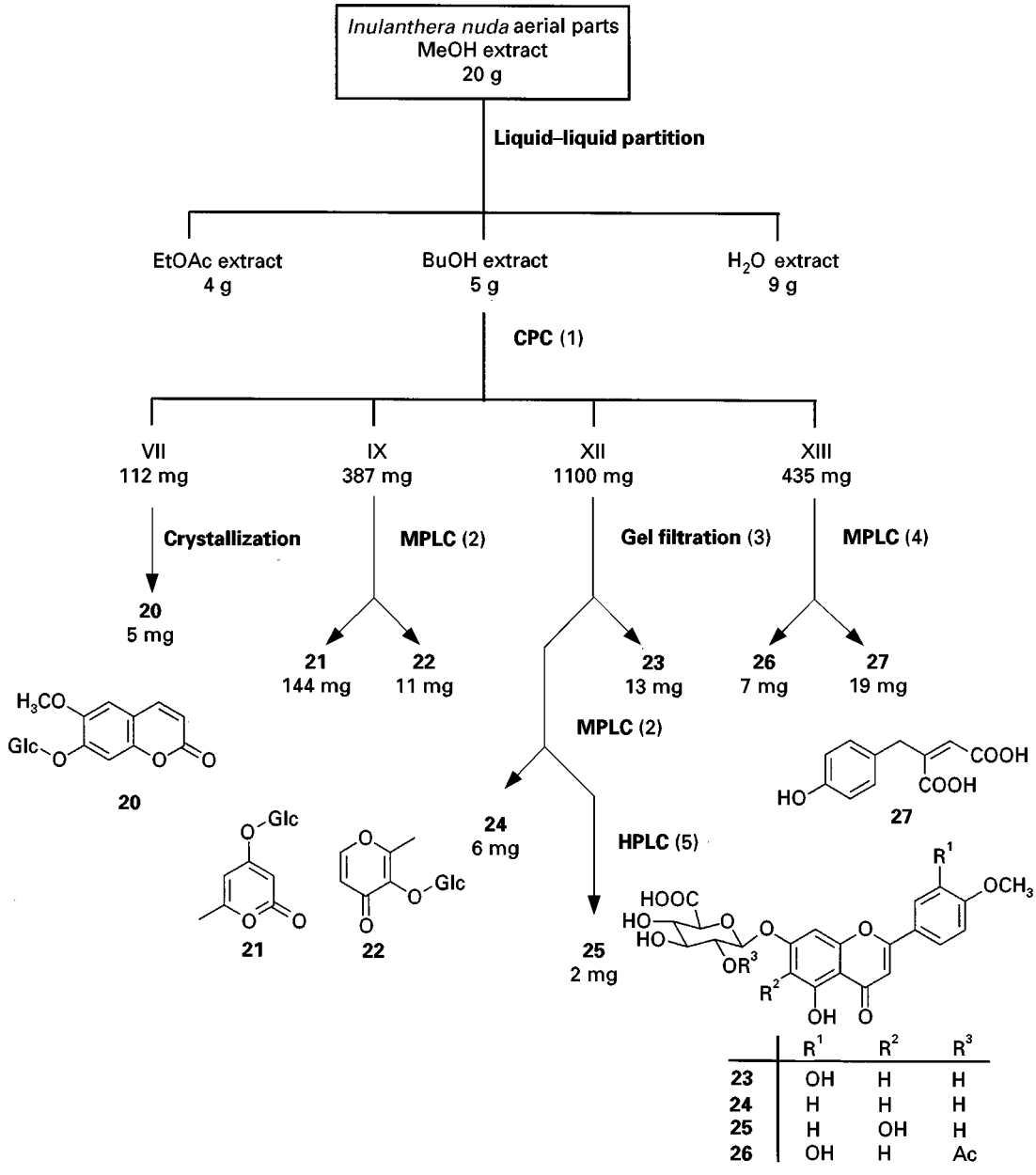

Figure 9 Strategy for the isolation of various aromatic constituents from *Inulanthera nuda* (Asteraceae). Conditions: 1, $CHCl_3$–MeOH–H_2O-*i*-PrOH; 2, RP-18, MeOH–H_2O (1 : 9); 3, Sephadex LH-20, MeOH–H_2O (1 : 1); 4, RP-18, MeOH–H_2O (1 : 4); 5, RP-18, MeCN–H_2O (7 : 43).

amounts of material, e.g. column chromatography using relatively cheap stationary phases (silica, alumina, polyamide or XAD ion exchange resins), flash chromatography or CCC. Size exclusion chromatography (SEC) is also becoming increasingly popular as a first purification step. Subsequent chromatographic steps on smaller quantities can be performed with more expensive column packings and equipment. Semipreparative HPLC is often reserved for final purification.

The combination of different preparative chromatography techniques for the isolation of various aromatic compounds from *Inulanthera nuda* (Asteraceae) is presented in **Figure 9**. A first liquid–liquid partition of the methanolic extract, dissolved in water, gave an enriched butanol extract which was further separated by CPC, affording 13 fractions. One of these fractions yielded (**20**) after recrystallization while the other constituents were further separated by MPLC or SEC. The final purification of flavonoid glycoside (**25**), for example, required a combination of SEC on Sephadex LH-20, followed by MPLC and semipreparative HPLC.

Conclusions

The introduction of modern liquid chromatographic methods has revolutionized the science of separation of natural products of plant origin. These new methods allow faster separations and facilitate the resolution of complex mixtures. As shown, techniques such as HPLC can be used both at the analytical and preparative level. At the analytical level and in combination with sophisticated detectors, structural information can be obtained online, while on the preparative scale closely related compounds can be successfully isolated.

The actual separation method or methods depend(s) on a number of factors relevant to the separation problem, but a judicial choice of strategy enables most targets to be reached. New methods and improvements are continually being introduced, with the result that the number of combinations available is steadily expanding, hopefully leading to a progressive simplification of the ever more complex separation problems that are being undertaken.

See also: **II/Chromatography: Liquid:** Detectors: Mass Spectrometry; Large-Scale Liquid Chromatography. **Extraction:** Solid-Phase Extraction. **III/Flash Chromatography. Medium-Pressure Liquid Chromatography. Natural Products:** High-Speed Countercurrent Chromatography; Thin-Layer (Planar) Chromatography. **Pigments:** Thin-Layer (Planar) Chromatography.

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Liquid Chromatography–Nuclear Magnetic Resonance

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Hyphenation of chromatographic and spectroscopic methods is important in analytical chemistry and is of great value in modern natural product analysis. Gas chromatography–mass spectrometry (GC-MS) has been used for many years to analyse volatile compounds and derivatives of nonvolatile natural products. The development of liquid chromatography–mass spectrometry (LC-MS) extended the scope of MS coupling techniques to allow analysis of nonvolatile compounds without derivatization. Nuclear magnetic resonance (NMR) is less sensitive than MS but represents the most informative and most universal analytical technique for natural products. Thus, using a NMR spectrometer in coupling methods does not simply mean adding another detector but represents a new dimension in analytical natural product chemistry.

The combination of NMR and chromatographic, or electrophoretic, separation methods was made